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Temporal Release of Fatty Acids and Sugars in the Spermosphere: Impacts on *Enterobacter cloacae*-Induced Biological Control[▽]

Sofia Windstam† and Eric B. Nelson*

Cornell University, Department of Plant Pathology and Plant-Microbe Biology, 334 Plant Science Building, Ithaca, New York 14853

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The aim of this study was to determine the temporal release of fatty acids and sugars from corn and cucumber seeds during the early stages of seed germination in order to establish whether sugars found in exudate can prevent exudate fatty acid degradation by *Enterobacter cloacae*. Both saturated (long-chain saturated fatty acids [LCSFA]) and unsaturated (long-chain unsaturated fatty acids [LCUFA]) fatty acids were detected in corn and cucumber seed exudates within 15 min after seed sowing. LCSFA and LCUFA were released at a rate of 26.1 and 6.44 ng/min/seed by corn and cucumber seeds, respectively. The unsaturated portion of the total fatty acid pool from both plant species contained primarily oleic and linoleic acids, and these fatty acids were released at a combined rate of 6.6 and 0.67 ng/min/seed from corn and cucumber, respectively. In the absence of seed exudate sugars, *E. cloacae* degraded linoleic acid at rates of 29 to 39 ng/min, exceeding the rate of total fatty acid release from seeds. Sugars constituted a significant percentage of corn seed exudate, accounting for 41% of the total dry seed weight. Only 5% of cucumber seed exudate was comprised of sugars. Glucose, fructose, and sucrose were the most abundant sugars present in seed exudate from both plant species. Corn seeds released a total of 137 μg/seed of these three sugars within 30 min of sowing, whereas cucumber seeds released 0.83 μg/seed within the same time frame. Levels of glucose, fructose, and sucrose found in corn seed exudate (90 to 342 μg) reduced the rate of linoleic acid degradation by *E. cloacae* to 7.5 to 8.8 ng/min in the presence of either sugar, leaving sufficient concentrations of linoleic acid to activate *Pythium ultimum* sporangia Our results demonstrate that elevated levels of sugars in the corn spermosphere can prevent the degradation of LCUFA by *E. cloacae*, leading to its failure to suppress *P. ultimum* sporangial activation, germination, and subsequent disease development.

The oomycete plant pathogen *Pythium ultimum*, which causes seed and seedling infections on many different plant species, is well adapted to growth and development in the spermosphere. *P. ultimum* has the capacity to rapidly switch from a quiescent state to active growth in the presence of seeds of a suitable host (16). Since *P. ultimum* spends the greater part of its life cycle as sporangia or oospores in a dormant state in soil, seed exudates are critical in triggering this developmental transition (17, 25).

Germination of *P. ultimum* sporangia in the spermosphere is initiated by molecules released from seeds within the first 15 to 30 min after sowing (27). With cotton seeds, long-chain unsaturated fatty acids (LCUFA) found in seed exudates are able to elicit this rapid sporangial germination response (24). Rapid mycelial growth and seed colonization follow sporangial germination (27), resulting in embryo infection within 24 to 48 h (7, 15, 28). Both sporangial germination and seed colonization are critical stages in *Pythium* pathogenesis, and if either is eliminated, disease will not develop (8, 12).

Seed infections caused by *P. ultimum* are reduced when seeds are coated with the gram-negative bacterium *Enterobacter cloacae* (10, 26). The mechanism by which *E. cloacae* achieves such control is by preventing the initiation of sporangial germination in the spermosphere within 1 h of sowing (27), thereby suppressing subsequent disease development. However, not all plants are protected by *E. cloacae*. For example, *E. cloacae* is extremely effective in protecting seeds of plants such as cotton and cucumber but fails to protect seeds of plants such as pea and corn (10) because of its failure to block sporangial activation and germination (27). One possible explanation for this failure is that other exudate molecules are released into the spermosphere that regulate the degradation of LCUFA by *E. cloacae*.

Genes within the *fad* regulon of gram-negative bacteria are responsible for uptake and degradation of both long-chain saturated fatty acids (LCSFA) and LCUFA from the environment (6). Many of the *fad* genes are transcriptionally induced in the presence of exogenous FA (3, 4). However, because FA are considered less attractive sources of carbon for sustaining bacterial growth and metabolism, these genes are under additional transcriptional regulation by the cyclic AMP receptor protein. If glucose is present in the environment or if the extracellular osmolarity is high, *fad* genes are not transcribed despite the presence of LCSFA and LCUFA (9, 22).

Seed exudates contain a wide spectrum of compounds, including FA and sugars (16), each of which is known to regulate expression of bacterial *fad* genes (3). It is conceivable that exudate sugars serve as regulators of *E. cloacae* FA degradation in the spermosphere, explaining the differential activity of *E. cloacae* in corn and cucumber spermospheres. The qualitative and quantitative changes in FA and sugar exudation within the first few minutes of sowing, the most critical window for *E. cloacae* activity in the spermosphere (27), and the influence of

* Corresponding author. Mailing address: Cornell University, Department of Plant Pathology, 334 Plant Science Building, Ithaca, NY 14853. Phone: (607) 255-7841. Fax: (607) 254-6448. E-mail: ebn1@cornell.edu.
† Present address: Michigan State University, Department of Plant Pathology, 140 Plant Biology Building, East Lansing, MI 48824.
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exudate sugars on *E. cloacae* LCUFA degradation are unknown (16). Based on these observations, the current study was designed to test the following two hypotheses: (i) the rate of LCUFA release from corn seeds greatly exceeds the rate of degradation by *E. cloacae*, and alternatively, (ii) corn seeds release sugars at the same time as elicitor FA and at concentrations sufficient to repress *E. cloacae* LCUFA degradation.

MATERIALS AND METHODS

Plant material. To reduce experimental variability, corn (*Zea mays* cv. Northern X-tra Sweet) and cucumber (*Cucumis sativus* cv. Marketmore) seeds were sorted by discarding cracked, deformed, or discolored seeds. Seeds were then surface sterilized for 3 min in 0.05% sodium hypochlorite with 1 to 2 drops of Tween 20, rinsed with sterile deionized water (sdw), and blotted dry. Because of the relationship between water uptake and seed exudation (20), we determined water uptake rates from individual seeds of corn and cucumber sown in 4 ml of washed sterile sand (0.5- to 1-mm particle size) or 4 ml sterile glass beads (0.1-mm particle size) in Corning 12-well tissue culture plates. sdw was added to each well to bring the final water content to 18% (wt/wt) (−2 kPa in sand). Additionally, imbibition rates were established for 3-g aliquots of seeds placed in water. All samples were incubated at 24°C. Seeds were harvested; rinsed; blotted dry; and weighed at 0.5, 1, 2, and 4 h after sowing. At least two replicate assays were performed for each plant species and matrix.

Production and germination of *Pythium ultimum* sporangia. *Pythium ultimum* isolate P4 was routinely grown on a mineral salts medium (SM+L) amended with 0.1% soy lecithin (α-phosphatidylcholine; Sigma, United States). Previous work demonstrated that sporangia produced on this media mimic those produced on living plant tissue (19). Six-millimeter-diameter agar discs were cut from 5-day-old cultures incubated at 27°C. These discs were leached twice in darkness for 10 min each, followed by a 3-h leaching with a buffer [0.01 M $Ca(NO_3)_2 \cdot 4H_2O$, 0.04 M $MgSO_4 \cdot 7H_2O$, 0.05 M KNO_3, pH 5.8]. The leaching buffer was replaced with fresh buffer at the end of each leaching period. Discs were rinsed twice with sdw and incubated in the dark for 2 days at 24°C. Ten microliters of seed exudate dissolved in 10 mM potassium phosphate buffer (pH 6.0) at concentrations ranging from 0 to 20 μg/μl or cell-free *E. cloacae*-treated solutions was added to leached discs. Discs were incubated for 3 h at 24°C and stained with 0.03% acid fuchsin in 85% lactic acid, and the numbers of germinated and nongerminated sporangia were enumerated at ×250 to ×400 by making an optical section across the entire diameter length of each disc.

Growth and maintenance of *E. cloacae*. *E. cloacae* strain EcCT501-R3 was grown on tryptic soy agar (Becton Dickinson, Cockeysville, MD) for 24 h. These cells were then used to inoculate 25 ml of tryptic soy broth (Becton Dickinson). Tryptic soy broth cultures were incubated for 18 h at 37°C on a rotary shaker at 130 rpm. Cells were collected by centrifugation (10 min at 5,000 rpm), the supernatant was discarded, and the pellet was washed twice with 0.1% sodium pyrophosphate buffer before being resuspended in M9 medium (14). Cell density was adjusted to 10^9 cells/ml by measuring the optical density at 600 nm.

Collection of seed exudate. Seeds were selected and surface sterilized as described above. Three grams of seeds was added to 30 ml of sdw in 125-ml flasks, and seed exudate was collected by shaking the flasks at 150 rpm at 24°C for 0.25, 0.5, 1, 2, 4, and 6 h. Exudate was harvested by decanting the solution through sterile cheesecloth into 50-ml tubes. The antioxidant butylated hydroxytoluene (Sigma, United States) was added to each tube at a final concentration of 10 ppm. Ten 10-μl aliquots of the exudate solution were spotted onto plates of potato dextrose agar (Difco, United States) and tryptic soy agar and assessed for contamination after 48 h. Noncontaminated solutions were pooled, lyophilized, and stored frozen under an atmosphere of argon. Four replicate flasks were prepared for each treatment. The exudate collection was repeated three times.

Extraction, derivatization, and analysis of seed exudate FA. Aqueous seed exudate solutions (30 ml) were spiked with 30 μg of tridecanoic acid as an internal control to assess extraction efficiency and acidified with sulfuric acid to a pH of ~3.5 before extraction. FA were extracted using conditioned 500 mg Oasis HLB solid-phase extraction columns (Waters Corp., Massachusetts) and using a two-dimensional elution protocol with chloroform-methanol (2:1, vol/vol) and methanol according to the manufacturer's instructions. All solvents used were high-pressure liquid chromatography grade. Samples were evaporated and redissolved in 2 ml methanol with 1% sulfuric acid and spiked with 30 μg of nonadecanoic acid as an internal control to assess esterification efficiency. Samples were vortexed and incubated at 85°C for 50 min. After incubation, 1.2 ml sdw was added and then methyl esters were extracted twice with 2 ml hexane. The organic fraction was dried over anhydrous granular sodium sulfate, evaporated, and redissolved in methylene chloride. Samples were injected (1 to 2 μl) into an HP5890 (Hewlett-Packard) gas chromatograph (GC), equipped with a DB-225 column (20-m length, 0.18-mm inner diameter, 0.2-μm film thickness) and a flame ionization detector (FID). The injector and detector temperatures were 230°C. The oven starting temperature was set at 150°C and held for 1 min, followed by an increase of the temperature to 240°C at 4°C/min, and the high temperature was held for 10 min. Peak areas and retention times were integrated with an HP3393A integrator. Three replicates were analyzed for each treatment, and the experiment was repeated twice. Subsequently, samples from the same seed and time were pooled and 2-μl aliquots were injected into an HP6890 GC equipped with a Supelco Equity 5 column (30-m length, 0.25-mm inner diameter, 0.25-μm film thickness) and an Agilent HP5973 mass spectrometer (MS) detector. The same parameters used above were employed for the GC-MS analysis. Peak areas and retention times were integrated using Agilent ChemStation software. FA were identified on the basis of retention times and mass spectrograms compared to a standard mix and a library of mass spectrograms. Individual peaks were quantified by comparing peak areas with those of the internal standards.

Seed exudate sugar derivatization and analysis. Seed exudate sugars were derivatized and analyzed as described by Roberts et al. (23). Briefly, 10- to 100-μl aliquots of 40-mg/ml exudate were lyophilized in 250-μl glass inserts placed in 2-ml autosampler vials. To each vial was added 25 to 50 μl *N*-methyl-bis(trifluoroacetamide) and 25 to 50 μl pyridine. Vials were capped with Teflon septa and heated at 65°C for 2 h with occasional vortexing. Two-microliter aliquots were injected into an HP5890 GC equipped with an Ultrawax 2 column (25-m length, 0.2-mm inner diameter, 0.33-μm film thickness) and a FID. The injector and detector temperatures were set to 250 and 300°C, respectively. The initial oven temperature of 75°C was held for 1 min, and the oven temperature was ramped at 10°C/min to a final temperature of 225°C and held for 2 min. Peak areas and retention times were integrated with an HP3393A integrator. Individual sugars were identified and quantified by comparing the retention time and peak areas to external standards. Standard curves were prepared using sugars that have been previously reported in seed exudates (16, 23). Increasing concentrations of each sugar were derivatized as described above, and 2-μl aliquots were used for GC analysis to determine retention times and peak areas. Regression equations were then calculated to establish relationships between absolute sugar levels and retention time. Three replicates were analyzed for each treatment, and the experiment was repeated twice.

Evaluation of LA degradation by *E. cloacae*. M9 medium containing either 200 or 350 μg/ml of sodium linoleate (Sigma Chemical Co., St. Louis, MO) dissolved in 1% Brij (Acros Organics, New Jersey) was used to evaluate the degradation of linoleic acid (LA) by *E. cloacae*. For sugar assays, M9 medium containing 200 μg/ml of LA was used as a *P. ultimum* sporangial germination stimulant. Glucose, fructose, or sucrose was added to the M9 medium to achieve final concentrations of 0, 1, 2, 3, 4, 5, and 10 mM, which equate to totals of 0, 18, 36, 54, 72, 90, and 180 μg of glucose and fructose and 0, 36, 72, 108, 144, 180, and 360 μg of sucrose per 100 μl, respectively. Each well of a 96-well microtiter plate was inoculated with 50 μl of *E. cloacae* cell suspension (final concentration of 5×10^8 cells/ml) and 50 μl of treatment solution. Negative controls consisted of 10 mM test sugar (180 μg/100 μl for both glucose and fructose and 360 μg/100 μl for sucrose) without LA to preclude the possibility of sugars eliciting germination responses. Previous studies have demonstrated that *P. ultimum* sporangia produced as described above do not germinate in response to glucose, fructose, or sucrose alone (19, 20). Plates were incubated at 27°C, and each treatment had three replicates.

The contents of the wells were transferred to 2-ml tubes after various incubation times and centrifuged. Well contents were removed at 0, 2, 4, and 8 h for the glucose and fructose assays; at 0, 4, 8, and 10 h for the sucrose assays; and at 0, 2, 4, 6, 8, 10.5, 12, and 24 h for the LA degradation assay (no sugars added). Supernatants were transferred to new tubes, and butylated hydroxytoluene was added to a final concentration of 10 ppm to reduce oxidation. Supernatants were then frozen under an atmosphere of argon. Experiments were repeated at least twice.

LA quantification. LA levels in samples from the glucose and sucrose assays were analyzed with GC. LA was extracted from sugar-amended M9 medium by using a liquid-liquid extraction procedure described by Folch et al. (5). A solution of chloroform and methanol (2:1, vol/vol) was added to the acidified samples (pH ~3.5) in an equal volume. The sample was vortexed for 1 min, and the organic phase was collected in a 2-ml glass vial. The extraction was repeated, and the organic phases were combined. Samples were then dried under a stream of nitrogen and redissolved in hexane. An online derivatization method was used in which 2 μl of bis(trimethylsilyl)trifluoroacetamide and 1 μl of sample were

coinjected into an HP5890 GC, equipped with an HP-255 column (25-m length, 0.2-mm diameter, 0.2-μm film thickness) and FID (21). The injector and detector temperatures were 230°C. Isothermal analyses (at 230°C) were conducted, and the total run time was 7 min. LA peak areas and retention times were integrated with an HP3393A integrator. Peak areas were quantified using an external LA standard. Three injections were made for each sample analyzed.

Statistical analysis. Exudate yields, sporangial germination rates, and individual FA and sugar components were analyzed with analysis of variance using the PROC GLM procedure in SAS v9.1 (SAS Institute, Cary, NC). Probit analysis using Minitab v14.2 (Minitab Inc., Pennsylvania) was used for estimating the 50% effective concentration (EC_{50}) values (exudate concentration that stimulates 50% sporangium germination). Means were separated using Student's *t* test, and least significant difference tests were used for pairwise comparisons. Additionally, simple linear regression was performed on time course studies (sugar and FA release, germination in response to *E. cloacae*-treated solutions, and FA degradation) followed by analysis of covariance for comparison of treatment slopes. The data from sugar assays (sporangium germination and remaining levels of LA) were analyzed by multiple regression (MR). First, a one-way analysis of variance was performed to ensure that the negative control was significantly different from all other treatments so that this treatment could be eliminated from the subsequent MR analysis. Germination data were transformed {arcsine [sqrt(*p*)] where *p* is the proportion of germinated sporangia} to stabilize the normality and variance when necessary. Diagnostic plots were performed for all the data to ensure that assumptions of the tests were fulfilled. Additionally, for MR analysis partial regression plots were analyzed to assess linearity between the response variable and each individual predictor variable, in the presence of all other variables. Insignificant terms ($\alpha > 0.05$) were dropped in all tested models.

RESULTS

Seed water uptake characteristics in different matrices and seed exudate yields. Both cucumber and corn seeds rapidly imbibed significant amounts of water within the first 30 min in all matrices tested. Water uptake continued to increase over the 4-h sampling period (Fig. 1). Both corn and cucumber seeds imbibed the most water when the seeds were placed in free water with no solid matrix. Differences in water uptake between seeds imbibed in water and those imbibed in glass beads were most pronounced for cucumber and evident within 30 min of sowing. By 2 h, cucumber seeds in free water imbibed 38% as much water as did seeds imbibed in glass beads (6.2 versus 3.8 mg/seed). In contrast, corn imbibition was similar in all three matrices (free water, sand, and glass beads) for the first 2 h, after which corn seeds in free water had the greatest amount of water uptake. Corn seeds in free water imbibed approximately 17% more water than did those imbibed in glass beads (52 versus 43 mg/seed). Although the absolute amount of water taken up by seeds in the various matrices differed, imbibition rates were all similar for both corn and cucumber (Fig. 1).

Corn and cucumber seeds differed dramatically in their exudation characteristics. Individual corn seeds released 384 μg of exudate within the first 15 min of imbibition compared to 42 μg released from cucumber during that same period (Table 1). By 6 h, over 1,800 μg exudate/seed was released from corn, representing approximately 1% of the initial weight of the seed. In contrast, exudates released within 15 min from cucumber seeds represent the bulk of the exudate released over the entire 6-h sampling period. By 6 h, cucumber seeds released only 68 μg exudate/seed (0.27% of initial seed weight), which did not differ significantly from the amount of exudate released within 15 min.

***P. ultimum* sporangial germination in response to seed exudates.** Corn seed exudate was significantly more stimulatory to sporangial germination than was cucumber seed exudate. The EC_{50} (exudate dose required to stimulate 50% sporangial germination) values for corn seed exudate were consistently lower than those for cucumber (Table 1). EC_{50} values ranged between 4.4 and 13.8 μg for corn and between 109.5 and 250.4 μg for cucumber. As a result, corn seed exudate was 18 to 25 times more stimulatory to *P. ultimum* sporangia than was cucumber exudate, depending on the time point sampled.

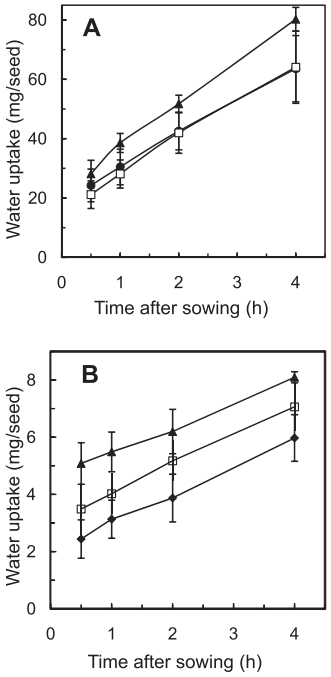

FIG. 1. Corn (A) and cucumber (B) water uptake in glass beads (● [A] and ◆ [B]), sand (□), and water (▲). Each marker represents the mean of at least six observations from at least two replicate experiments, and the error bars represent the standard deviations. Note the different *y* axes.

Qualitative and quantitative analysis of FA in seed exudates. Both corn and cucumber seeds released significant levels of FA within 15 min of initiating imbibition (Table 1). Although corn released four to seven times more FA than did cucumber over 6 h of germination, the release of FA by cucumber represented a greater proportion of the total exudate. Total FA (wt/wt) was equivalent to 0.11 to 0.32% of the total weight of corn seed exudate and 0.40 to 0.66% of the total weight of cucumber seed exudate (data not shown). Levels of FA did not increase significantly for either plant species beyond levels detected by 15 min.

The most abundant LCUFA detected in corn and cucumber seed exudate were oleic acid ($C_{18:1}$) and LA ($C_{18:2}$). By 15 min after sowing, corn seeds released 92 ng of $C_{18:1}$ and 83 ng of $C_{18:2}$ per seed (Fig. 2), which corresponds to a combined exudation rate of 11.6 ng/seed/min. Over the same 15-min period, cucumber exudate released 7 ng of $C_{18:1}$ and 41 ng of $C_{18:2}$ per seed, which equates to a combined exudation rate of 3.2 ng/seed/min. Within the first 6 h of imbibition, corn seeds had released 870 ng of $C_{18:1}$ and 255 ng of $C_{18:2}$ per seed. The release of these two FA from cucumber seeds fluctuated over time but did not increase significantly by 6 h. Trace amounts of

TABLE 1. Exudate, FA, and sugars released by corn and cucumber during the first 6 h of seed imbibition and the stimulatory activity of exudates at each collection time toward *Pythium ultimum* sporangial germination

Collection time (h)	Corn					Cucumber				
	Mean yield/seed ± SD				EC_{50}[d] (μg)	Mean yield/seed ± SD				EC_{50}[d] (μg)
	Exudate (μg)[a]	Exudate (mg/g)[a]	FA (μg)[b]	Sugars (μg)[c]		Exudate (μg)[a]	Exudate (mg/g)[a]	FA (μg)[b]	Sugars (μg)[c]	
0.25	384 ± 28	2.30 ± 0.17	1.22 ± 0.588	93.7 ± 49.1	13.8 ± 0.3	42 ± 20	1.67 ± 0.79	0.28 ± 0.15	0.74 ± 0.15	250.4 ± 1.6
0.5	493 ± 57	2.96 ± 0.34	0.94 ± 0.371	202 ± 73.4	12.8 ± 0.3	50 ± 6	2.02 ± 0.23	0.25 ± 0.14	2.1 ± 1.1	174.8 ± 6.6
1	739 ± 106	4.43 ± 0.64	1.04 ± 0.433	167 ± 84.0	12.4 ± 0.2	49 ± 6	1.98 ± 0.26	0.22 ± 0.05	2.1 ± 0.36	131.2 ± 3.2
2	1,001 ± 65	6.01 ± 0.39	1.81 ± 1.28	350 ± 82.6	8.9 ± 0.2	63 ± 20	2.50 ± 0.79	0.25 ± 0.05	2.5 ± 0.55	128.7 ± 3.0
4	1,399 ± 271	8.40 ± 1.62	1.59 ± 0.422	501 ± 143	7.3 ± 0.2	72 ± 28	2.88 ± 1.11	0.32 ± 0.09	ND[e]	128.7 ± 2.7
6	1,844 ± 259	11.07 ± 1.55	2.65 ± 2.48	729 ± 224	4.4 ± 0.2	68 ± 11	2.71 ± 0.43	0.37 ± 0.18	3.4 ± 1.1	109.5 ± 2.0

[a] Total exudate ($n = 3$).
[b] Total FA and total sugars ($n = 6$).
[c] Total sugars ($n = 6$).
[d] Mean EC_{50} (amount of exudate required to induce 50% sporangial germination as estimated by probit dose-response regression) ($n = 9$) ± standard error.
[e] ND, not determined.

myristoleic ($C_{14:1}$) and palmitoleic ($C_{16:1}$) acid were also detected inconsistently in cucumber seed exudate after 4 h of sowing at amounts of <3 ng/seed (data not shown).

Medium-chain saturated FA and LCSFA were the predominant FA detected in both corn and cucumber exudates. LCSFA comprised approximately 70 to 80% of all FA detected. The most abundant LCSFA found in exudates from both plant species were palmitic ($C_{16:0}$), stearic ($C_{18:0}$), and lauric ($C_{12:0}$) acids (Fig. 3). Lesser amounts of caprylic (8:0), capric (10:0), myristic (14:0), and behenic (22:0) acids were also detected, but these rarely represented more than 10% of total FA (levels combined and shown in Fig. 3). Azaleic acid, a dicarboxylic acid, was also detected at extremely low levels in both corn and cucumber seed exudate (data not shown).

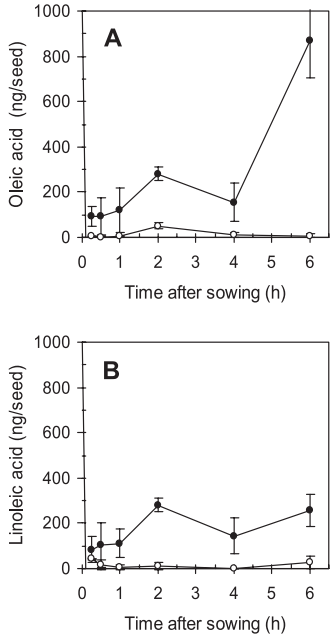

FIG. 2. Oleic acid (A) and LA (B) released from germinating corn (●) and cucumber (○) seeds during the first 6 h after sowing. Each marker represents the mean of six observations.

Qualitative and quantitative analysis of sugars in seed exudates. Corn seed exudates contained much higher levels of sugars than did cucumber exudates. By 15 min after the start of imbibition, corn seeds released 94 μg of sugars per seed whereas cucumber seeds had released only 0.7 μg/seed (Table 1). This increased to 729 μg/seed and 3.4 μg/seed, respectively, by 6 h. This represents 214 times more total sugars in corn seed exudates than in cucumber exudates over the 6-h sampling period. Sugars accounted for up to 41% of the total corn seed exudate on a weight basis but less than 5% of the total cucumber seed exudate.

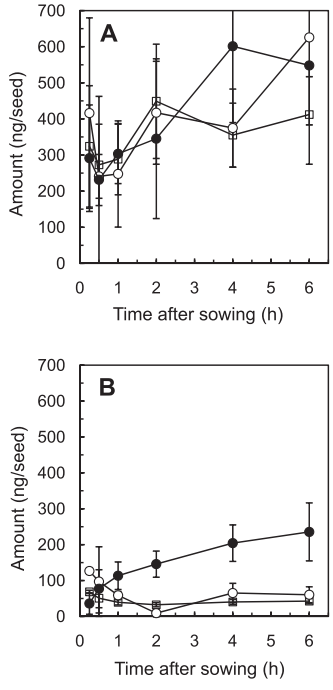

FIG. 3. Saturated FA (palmitic acid [□], stearic acid [○], and others [●]) released from germinating corn (A) and cucumber (B) seeds during the first 6 h after sowing. Other saturated FA include caprylic (8:0), capric (10:0), lauric (12:0), myristic (14:0), and behenic (22:0) acids. Each marker represents the mean of six observations.

TABLE 2. Release rates of long-chain FA and glucose, fructose, and sucrose from corn and cucumber seeds within the first 30 min of germination

Seed	FA release (ng/min/seed)		Sugar release (μg/seed)			
	Total[a]	OA[b] + LA	Glucose	Fructose	Sucrose	Total[c]
Corn	26.1	6.6	60.1	36.1	40.8	137.0
Cucumber	6.44	0.67	0.02	0.08	0.04	0.83

[a] Rate of release of all detected and quantified FA with carbon chains equal to or longer than C_{16}.
[b] OA, oleic acid.
[c] Total combined amount of glucose, fructose, and sucrose.

Glucose, fructose, and sucrose were the most abundant sugars detected in corn and cucumber seed exudates at all collection times. Other sugars detected at low levels in both corn and cucumber seed exudate included arabinose, cellobiose, galactose, lactose, maltose, mannitol, rhamnose, ribose, trehalose, and xylose. In addition, mannose and lactose were detected in cucumber exudate. The combined levels of glucose, fructose, and sucrose accounted for 71 to 83% and 37 to 60% of all the exudate sugars from corn and cucumber, respectively. Glucose was the most abundant sugar detected in corn exudate, accounting for 31 to 46% of all sugars released and comprising nearly 10% of the total dry weight of exudate released within 15 min of starting imbibition (Table 1). By 30 min of imbibition, 60.1 μg of glucose and a combined total of 137 μg of fructose, glucose, and sucrose was released by individual corn seeds (Table 2; Fig. 4A). Fructose was the most abundant sugar detected in cucumber seed exudate and comprised between 24 and 37% of all exudate sugars. Most of the fructose (0.780 μg/seed) was released from cucumber seeds within the first 30 min of imbibition, making up the bulk of the combined 0.827 μg of fructose, glucose, and sucrose released at that time point (Table 2; Fig. 4B).

Release of all sugars from corn seeds increased linearly over time ($P < 0.001$, data not shown). However, the rates of fructose and sucrose release did not differ but were significantly lower than the rate of glucose release (data not shown). There was no strong linear increase in sugar release from cucumber over the 6-h sampling period (data not shown). The bulk of all cucumber sugars were released during the first 30 min to 1 h of imbibition, after which no further increases were observed (Fig. 4B).

Germination response of *P. ultimum* sporangia to LA exposed to *E. cloacae*. The degradation and sporangial stimulation of LA in the presence of *E. cloacae* were studied over a 24-h period (Fig. 5). Lower percentages of LA-induced germinated sporangia were observed in the presence than in the absence of *E. cloacae*. Significant reductions in sporangial germination were observed by 4 h of incubation in the presence of 200 μg/ml LA, and by 12 h, only 7% sporangial germination remained. By 24 h of incubation, the level of remaining LA elicited less than 4% sporangial germination. As measured by sporangial germination levels, LA degradation rates were estimated to range from 23.8 to 25.8 ng/min.

Degradation of LA by *E. cloacae* in the presence of exudate sugars. Glucose, fructose, and sucrose, the most abundant sugars in both corn and cucumber seed exudate, each reduced the degradation of LA by *E. cloacae* (Fig. 6). The sporangial germination response to *E. cloacae*-treated LA-sugar solutions increased linearly with increasing sugar concentration. Additionally, the rate of decrease in sporangium germination in response to *E. cloacae*-treated LA solutions over time was significantly reduced when glucose or sucrose was added in excess of 54 and 108 μg, respectively (Table 3). No significant

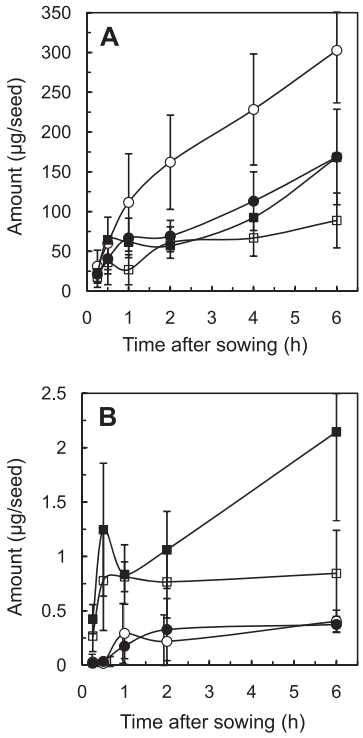

FIG. 4. Glucose (○), fructose (□), sucrose (●), and other sugars (■) released by germinating corn (A) and cucumber (B) seeds during the first 6 h after sowing. Other sugars include arabinose, cellobiose, galactose, lactose, maltose, mannitol, mannose, raffinose, rhamnose, ribose, trehalose, and xylose for corn. The same sugars except lactose and mannose are included in the same category for cucumber. Each marker represents the mean of six observations. Note the different *y* axes.

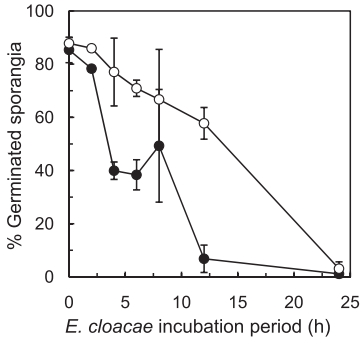

FIG. 5. Germination of *P. ultimum* sporangia in response to cell-free LA solutions that have been treated with *E. cloacae*. *E. cloacae* cells were incubated in 200-μg/ml (●) or 350-μg/ml (○) solutions of LA for 24 h. Cells were then removed, and the supernatant was assayed. Each marker represents the mean of three observations ± standard deviation.

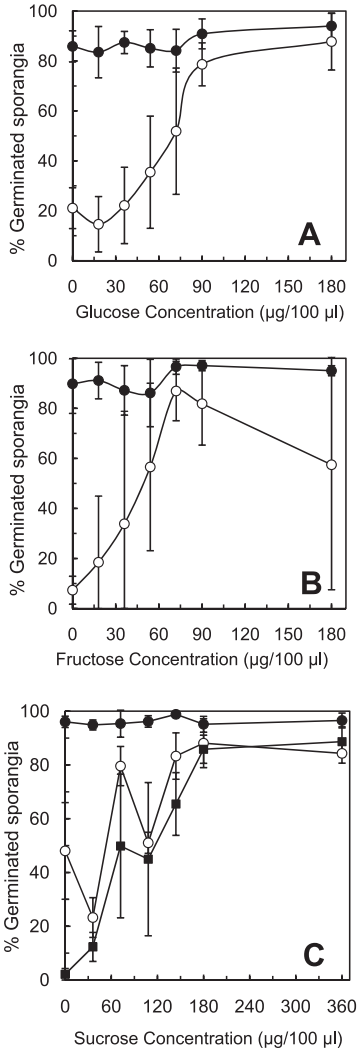

FIG. 6. Germination of *P. ultimum* sporangia in response to cell-free LA solutions amended with different concentrations of glucose (A), fructose (B), or sucrose (C) that have been treated with *E. cloacae* for 0 (●), 4 (○), or 10 (■) h. Each marker represents the mean of three observations ± standard deviation.

($P < 0.0001$) germination of sporangia was observed in the presence of sugars alone (no LA), indicating that sugars alone are not responsible for the sporangial germination observed (data not shown).

Incubating *E. cloacae* with LA in the absence of sugar reduced the percentage of germinated sporangia from 90% to 7% in as little as 4 h (Fig. 6A). This level of germination was significantly lower than that in LA samples containing 54 μg/ml of fructose or greater, where the sporangial germination percentages were greater than 57%. Differences in germination among LA solutions containing lower concentrations of fructose were not significant due to a high level of variability.

In the presence of glucose, *E. cloacae* degradation of LA after 4 h of incubation displayed a pattern similar to that in the presence of fructose (Fig. 6A). Significantly lower levels of sporangial germination were observed when no sugar was added to LA solutions than in the presence of 90 or 180 μg/ml glucose. Remaining levels of LA in these treatments resulted in sporangial germination rates of 79% and 88%, respectively.

In the presence of sucrose, however, the degradation of LA by *E. cloacae* resulted in a slightly different response (Fig. 6C). *E. cloacae* required a 10-h incubation period with sucrose before a pattern similar to that described above for glucose and fructose could be observed. There was significantly less sporangial germination induced by LA in the absence of sucrose after 10 h of incubation than with treatments containing 72 to 360 μg of sucrose. With all of the sugars tested, 72 to 180 μg was sufficient to significantly reduce the degradation of LA as measured by *P. ultimum* sporangial germination.

Levels of LA in the presence of glucose and sucrose were quantified after various periods of incubation with *E. cloacae* (Fig. 7). Degradation of LA by *E. cloacae* was reduced even at very low concentrations of each tested sugar. As glucose or sucrose concentrations increased, there was a significant ($P < 0.0001$) corresponding linear increase in the levels of LA remaining in samples, despite the presence of *E, cloacae*. Based on GC analyses of LA solutions incubated in the presence of *E. cloacae* (8 to 10 h) but in the absence of sugars, the rates of LA degradation by *E. cloacae* were between 29.2 and 39.2 ng LA/min (Table 3). Ninety micrograms of glucose and 180 μg of sucrose were the minimum amounts that significantly decreased the rate of LA degradation, and the presence of glucose and sucrose at 180 and 360 μg, respectively, reduced the rate of LA degradation to 8.8 and 7.5 ng/min, respectively.

DISCUSSION

This study was aimed at explaining why *E. cloacae* fails to reduce sporangial germination and subsequent disease development in the corn spermosphere while remaining fully suppressive to *Pythium* seed infections in the cucumber spermosphere. Two hypotheses were tested to explain this observation: (i) the rate of FA exudation from corn seeds exceeds the rate of FA degradation by *E. cloacae*, and (ii) seeds release sugars that repress FA degradation by *E. cloacae* in the spermosphere. If the rate of FA exudation from corn seeds

TABLE 3. Rate of LA degradation by *E. cloacae* in the presence of glucose or sucrose

Sugar concn [μg/100 μl (mM)][a]	LA degradation rate (ng/min)[b]	Regression coefficient (r^2)[c]
Glucose		
0 (0)	29.2 a	0.928***
18 (1)	33.7 a	0.788***
90 (5)	14.3 b	0.555***
180 (10)	8.8 b	0.331**
Sucrose		
0 (0)	39.2 a	0.905***
36 (1)	26.5 a	0.808***
180 (5)	12.3 b	0.435**
360 (10)	7.5 b	0.189*

[a] Solutions of M9 amended with glucose or sucrose in a total volume of 100 μl were treated with *E. cloacae* for up to 10 h.

[b] Rate of LA degradation by *E. cloacae*. Analysis of covariance was used to compare the slopes of treatments. Estimates with different letters are significantly different.

[c] Significance level of the F-test of the association between the level of remaining LA and time. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

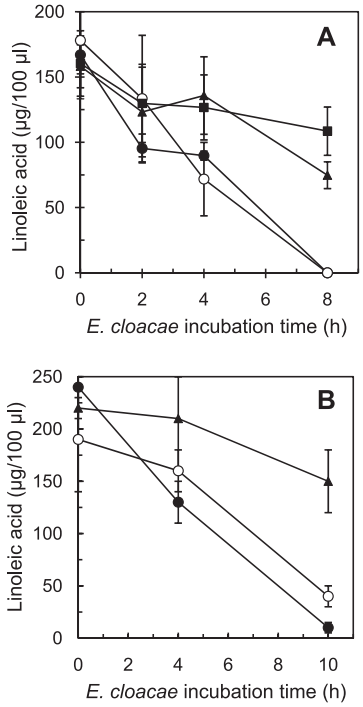

FIG. 7. Levels of remaining LAs (as measured by GC with a FID) in solutions amended with 0 (●), 18 (○), 90 (▲), or 180 (■) μg glucose/100 μl (A) or 0 (●), 36 (○), or 180 (▲) μg sucrose/100 μl (B) that have been incubated with *E. cloacae* for various periods of time. Each bar represents the mean of three observations ± standard deviation.

were to exceed the rate of FA degradation by *E. cloacae*, concentrations of LCUFA sufficient to stimulate the germination of *P. ultimum* sporangia could remain in the spermosphere and disease development would proceed normally. Alternatively, seeds are known to release sugars that are known to repress FA degradation in *Escherichia coli* and other gram-negative bacteria (2, 3, 23). If this were to occur with *E. cloacae* in the corn spermosphere, concentrations of LCUFA sufficient to elicit *P. ultimum* sporangial germination would escape bacterial degradation and again allow disease development to proceed normally.

Critical to the tests of these hypotheses is the characterization of FA and sugars released into the spermosphere within the first 30 to 60 min of sowing. If *E. cloacae* is to compete with *P. ultimum* sporangia for LCUFA, such competition must occur during this time frame since *E. cloacae* does not inhibit sporangial germination and disease development once sporangial activation has taken place (27). The results of our study reveal that, within this critical time frame, the rate of degradation of exudate FA by *E. cloacae* exceeds the rate at which they are released from seeds by nearly fourfold. Therefore, the first hypothesis can be rejected.

Previous studies have shown that many different sugars are released from seeds during germination (16). Roberts et al. (23) found that glucose, sucrose, and fructose were the most abundant sugars found in the seed exudates of cucumber and corn as well as other plant species within the first 96 h of seed germination. However, the concentrations within the first 30 to 60 min of germination were not determined. If sugar repression of *E. cloacae* FA degradation explains the failure of *E. cloacae* to reduce sporangial germination and subsequent disease development, then sufficient concentrations of sugars must be released during this critical time frame. Furthermore, the sugars must be those capable of repressing FA degradation. Our results confirm that detectable concentrations of glucose, sucrose, fructose, and other sugars are present during these early stages of seed germination for both cucumber and corn seeds. Although concentrations of sugars released from corn seeds exceeded those necessary to reduce FA degradation by *E. cloacae*, sugar concentrations in cucumber seed exudates were over 100 times lower than those required to reduce FA degradation. It is likely, therefore, that the lack of FA degradation by *E. cloacae* in corn seed exudates can explain the observed reduction in disease development, since the addition of glucose or fructose to cucumber and other low-sugar-exudation seeds is known to abolish the protection of seeds by *E. cloacae* against *P. ultimum* infection (18).

It is important to note that rates of exudation of FA and sugars as well as the rates of FA degradation were determined in a liquid matrix where seeds may display a different exudation rate than they would in a solid matrix. Although the rates of water imbibition (and hence exudation rate) did not differ among the three exudate collection systems used (glass beads, sand, and water), differences were observed in the total amount of water imbibed. Seeds imbibed more water when placed in free water; the lowest amount of water was taken up in glass beads. Since increased water uptake is accompanied by increased exudation during these initial stages of seed germination (16), it is possible that estimated rates of LCUFA and sugar release from corn and cucumber seeds are slightly inflated. Overestimation of exudate release would affect primarily the calculated values for cucumber seeds because they imbibed up to 38% more water when germinated in free water than when germinated in a solid matrix. However, for corn the total amount of water imbibed differed significantly between the solid and liquid matrices only after 2 h of sowing. Ultimately, this overestimation does not affect the overall conclusion that the rates of LCUFA degradation by *E. cloacae* either equal or exceed the rate of LCUFA release from seeds and that sugars released by corn seeds are sufficient to decrease LCUFA degradation.

Results from studies of root exudation dynamics also suggest that exudation rates may be underestimated under gnotobiotic conditions (11, 13), in part because the soil microbiota are known to increase not only the total level of exudation but also the composition of specific exudate components. Currently, no data are available on the effects of the soil microbiota on seed exudation. Considering that the initial release of exudates from seeds is a passive process (1), the lack of microorganisms during exudate collections would not be expected to have a significant impact on seed exudation dynamics because of the limited (60-min) experimental time frame.

Results from this study have validated the important role of FA competition in the spermosphere as an essential mechanism by which plant-associated bacteria interact with seed-infecting oomycete pathogens of plants. A key conceptual element to emerge from this study is the importance of understanding the synchronization of developmental behaviors

and activities of plant-associated microbes with specific spermosphere molecules that regulate those behaviors in understanding the nature of plant-associated microbial interactions. This is particularly critical in plant-associated habitats such as the spermosphere where the biochemical and microbial environment changes rapidly. Such synchronization provides a critical ecological context in which meaningful hypotheses can be tested. Continued studies of spermosphere habitats are likely to reveal important new insights into the nature of plant-associated microbial interactions.

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